A Role for Ubiquitination in Mitochondrial Inheritance in *Saccharomyces cerevisiae*

Harold A. Fisk and Michael P. Yaffe

University of California, San Diego, Department of Biology, La Jolla, California 92093

Abstract. The *smm1* mutation suppresses defects in mitochondrial distribution and morphology caused by the *mdm1-252* mutation in the yeast *Saccharomyces cerevisiae.* Cells harboring only the *smm1* mutation themselves display temperature-sensitive growth and aberrant mitochondrial inheritance and morphology at the nonpermissive temperature. *smm1* maps to *RSP5*, a gene encoding an essential ubiquitin-protein ligase. The *smm1* defects are suppressed by overexpression of wild-type ubiquitin but not by overexpression of mutant ubiquitin in which lysine-63 is replaced by arginine. Furthermore, overexpression of this mutant ubiquitin perturbs mitochondrial distribution and morphology in wild-type cells. Site-directed mutagenesis revealed that the ubiquitin ligase activity of Rsp5p is essential for its function in mitochondrial inheritance. A second mutation, *smm2*, which also suppressed *mdm1-252* defects, but did not cause aberrant mitochondrial distribution and morphology, mapped to *BUL1*, encoding a protein interacting with Rsp5p. These results indicate that protein ubiquitination mediated by Rsp5p plays an essential role in mitochondrial inheritance, and reveal a novel function for protein ubiquitination.

Key words: mitochondria • ubiquitin • yeast • organelle inheritance • cytoskeleton

Mitochondria propagate by growth and division of preexisting mitochondria (Palade, 1983; Attardi and Schatz, 1988). Therefore, an essential component of cell proliferation is the distribution of mitochondria to daughter cells before cytokinesis. In the yeast *Saccharomyces cerevisiae*, mitochondrial inheritance involves the vectorial transfer of mitochondria from the mother portion of the cell into the developing daughter bud (Stevens, 1981). Cellular components required for this process have been identified through the analysis of mutant yeast strains that display defects in mitochondrial distribution and morphology, the *mdm* mutants (McConnell et al., 1990; Berger and Yaffe, 1996). These studies have revealed the importance of three integral proteins of the mitochondrial outer membrane (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997), as well as key functions for several cytoplasmic proteins (McConnell and Yaffe, 1992; Hermann et al., 1997), but additional components and basic underlying mechanisms remain to be identified.

Mitochondrial inheritance in budding yeast requires Mdm1p, an intermediate filament-like protein localized to a series of punctate structures distributed throughout the cytoplasm (McConnell and Yaffe, 1992). The distribution, stability, and apparent composition of these structures suggest that they function as part of a cytoskeleton-like system that mediates mitochondrial and nuclear positioning (McConnell and Yaffe, 1992, 1993). In *mdm1-1* mutant cells at the nonpermissive temperature, these punctate structures disassemble and mitochondrial transmission to buds is defective (McConnell and Yaffe, 1992). Microscopic analysis of *mdm1-1* mutant cells has also revealed a role for Mdm1p in the transmission of nuclei to daughter buds, and *mdm1-1* cells display defects in orientation of the mitotic spindle (McConnell and Yaffe, 1992; Fisk and Yaffe, 1997). These functions of Mdm1p in mitochondrial and nuclear inheritance have been uncoupled in a series of additional mutant *mdm1* alleles that cause defects exclusively in mitochondrial or nuclear inheritance (Fisk and Yaffe, 1997).

The *mdm1-252* mutation causes mitochondrial inheritance defects but has no effect on nuclear segregation (Fisk and Yaffe, 1997). To identify proteins that interact with Mdm1p as part of its function in mitochondrial inheritance, second-site suppressors of *mdm1-252* were isolated. Analysis of two of these suppressors has revealed a role for the ubiquitin-protein ligase, Rsp5p, in mitochondrial inheritance.

Address correspondence to Michael P. Yaffe, Department of Biology, 0347, University of California, San Diego, La Jolla, CA 92093-0347. Tel.: (619) 534-4769. Fax: (619) 534-4403. E-mail: myaffe@ucsd.edu

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Table I. Yeast Strains

Strain	Genotype	Source
MYY290	MAT a, MDM1, *his3, leu2, ura3*	Smith and Yaffe, 1991
MYY291	MAT α, MDM1, *his3, leu2, ura3*	Smith and Yaffe, 1991
MYY298	MAT a/α, MDM1/MDM1, *his3/his3, leu2/leu2, ura3/ura3*	Smith and Yaffe, 1991
MYY403	MAT α, *mdm1-1, leu2, ura3*	McConnell and Yaffe, 1992
MYY700	MAT a, *mdm1-20, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY701	MAT a, *mdm1-199, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY702	MAT a, *mdm1-200, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY704	MAT a, *mdm1-202, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY709	MAT a, *mdm1-204, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY710	MAT a, *mdm1-217, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY715	MAT a, *mdm1-227, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY717	MAT a, *mdm1-228, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY720	MAT a, *mdm1-251, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY721	MAT a, *mdm1-252, his3, leu2, ura3*	Fisk and Yaffe, 1997
MYY803	MAT a, *mdm1-252, smm1, his3, leu2, ura3*	This study
MYY804	MAT a, *mdm1-252, smm2, his3, leu2, ura3*	This study
MYY805	MAT a, *mdm1-252, smm3, his3, leu2, ura3*	This study
MYY806	MAT a, *mdm1-252, smm4, his3, leu2, ura3*	This study
MYY808	MAT a, *MDM1, smm1, his3, leu2, ura3*	This study
MYY809	MAT α, *MDM1, smm1, his3, leu2, ura3*	This study
MYY812	MAT α, *MDM1, smm2, his3, leu2, ura3*	This study
MYY813	MAT α, *MDM1, smm 2, his3, leu2, ura3*	This study
MYY820	MAT α, *MDM1, RSP5::pRS305-RSP5, his3, leu2, ura3*	This study
MYY823	MAT α, *MDM1, smm1, HIS3, leu2, ura3*	This study
MYY825	MAT a/α, *MDM1/MDM1, RSP5/rsp5::HIS3, his3/his3, leu2/leu2, ura3/ura3*	This study
MYY826	MAT a, *MDM1, Δrsp5::HIS3, his3, leu2, ura3,* pRS316-RSP5	This study
MYY829	MAT a, *MDM1, Δrsp5::HIS3, his3, leu2, ura3,* pRS316-smm1	This study
MYY832	MAT a, *MDM1, Δrsp5::HIS3, his3, leu2, ura3,* pRS316-mdp1-1	This study
MYY833	MAT a, *MDM1, Δrsp5::HIS3, his3, leu2, ura3,* pRS316-mdp1-13	This study
MYY834	MAT a, *MDM1, Δrsp5::HIS3, his3, leu2, ura3,* pRS316-mdp1-14	This study
LHY1	MAT a, *his4, leu2, ura3, lys2, bar1-1*	L. Hicke
LHY180	MAT a, *ubc1::HIS3, ura3, leu2, his3, trp1, lys2, bar1-1*	L. Hicke
LHY192	MAT a, *ubc4::TRP1, ura3, leu2, his3, trp1, lys2, ade2, bar1-1*	L. Hicke
LHY201	MAT a, *ubc5::LEU2, his3, his4, leu2, ura3, lys2, trp1, bar1-1*	L. Hicke
LHY183	MAT a, *ubc1::HIS3, ubc4::TRP1, his3, leu2, ura3, lys2, trp1, bar1-1*	L. Hicke
LHY21	MAT a, *ubc4::TRP1, ubc5::LEU2, his3, his4, leu2, ura3, ade2, lys2, trp1, bar1-1*	L. Hicke

Materials and Methods

Yeast Strains and Genetic Methods

S. cerevisiae strains used in this study are listed in Table I. Strains MYY290, MYY291, and MYY298 (Smith and Yaffe, 1991), MYY403 (McConnell and Yaffe, 1992), MYY535 (Nickas and Yaffe, 1996), and MYY700-MYY721 (Fisk and Yaffe, 1997) have been described previously. Strains MYY803 and MYY804 were isolated as pseudorevertants of *mdm1-252* as described below. Strains MYY808 and MYY809 were isolated from a backcross of strain MYY803 to strain MYY291. Strains MYY812 and MYY813 were isolated from a backcross of strain MYY803 to MYY291. Strain MYY820, a *MAT***a** strain marked with *LEU2* at the *RSP5* locus, was created as described below. Strain MYY823 was created by crossing strain MYY809 to strain X21801A (Yeast Genetics Stock Center), sporulating the resulting diploid, and isolating a temperature-sensitive, Ura^-, Leu^-, haploid spore. Strain MYY825 was created by disrupting one copy of *RSP5* with *HIS3* in MYY298, as described below. Strains MYY816 and MYY817 in which *BUL1* is replaced by *LEU2* were generated as described below. Strains MYY826, MYY829, MYY832, MYY833, and MYY834 were generated by transformation of MYY825 with plasmids pRS316-RSP5, pRS316-smm1, pRS316-mdp1-1, pRS316-mdp1-13, pRS316-mdp1-14, respectively, sporulation of transformed strains, and recovery of His^+, Ura^+, haploid spores. Strains LHY1 (RH448), LHY180 (RH3136), LHY192 (RH3132), LHY201 (RH3096), LHY183 (RH3147), and LHY21 (RH3097) were obtained from Linda Hicke (Northwestern University) and have been described previously (Hicke and Riezman, 1996). Media and genetic analyses were as described previously (Rose et al., 1990).

Isolation of Second Site Suppressors of mdm1-252

Second site suppressors were identified by pseudoreversion analysis. Approximately 5×10^8 MYY721 (*mdm1-252*) cells were plated at a density of $0.5–1 \times 10^6$ cells per plate onto yeast extract/peptone/glucose (YPD)[1]-agar medium at 37°C. Cells were replica-plated every day for 3 d onto prewarmed YPD-agar plates at 37°C. Clones able to grow as serial replica colonies at 37°C were tested for the ability to grow as single isolated colonies at 37°C. Mitochondrial distribution and morphology of apparent revertant colonies were analyzed by staining with the mitochondria-specific vital dye 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI) as described previously (McConnell and Yaffe, 1992). To determine if pseudorevertants harbored second site suppressing mutations, candidate strains were backcrossed to strain MYY291 and meiotic progeny were analyzed for temperature-sensitive growth. Backcrossing also revealed whether suppressing mutations caused temperature-sensitive growth defects in the presence of wild-type *MDM1.*

Phenotypic Analysis

Mitochondrial inheritance in living cells was analyzed by DASPMI stain-

1. *Abbreviations used in this paper:* CIP, calf intestinal phosphatase; DAPI, 4,6-diamidino-2-phenylindole; DASPMI, 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide; YPD, yeast extract/peptone/glucose.

ing of cells grown in liquid cultures as described previously (McConnell and Yaffe, 1992). Cellular distribution of Mdm1p structures, mitochondrial outer membranes, and microtubules was examined by indirect immunofluorescence microscopy as described previously (Fisk and Yaffe, 1997). Nuclear and mitochondrial DNAs were visualized by fluorescence microscopy after staining with 4,6-diamidino-2-phenylindole (DAPI) (McConnell and Yaffe, 1992; Williamson and Fennell, 1975).

Cloning of smm1 and smm2

Strain MYY823 was transformed with two different genomic libraries in the centromere-based vectors YCp50 (Rose et al., 1987) and pSB32 (Rose and Broach, 1991). Four plasmids capable of completely complementing the temperature-sensitive growth of *smm1* were isolated: YCp50-3.1 and YCp50-3.10 from the Rose library and pSB-8 and pSB-16 from the pSB32-based library. DNA sequence was determined for each end of the genomic DNA inserts in these plasmids using the pBR322 BamHI cw and BamHI ccw sequencing primers (Promega). DNA sequences of plasmid inserts were compared to the *Saccharomyces* Genome Database using the BLAST program (Altschul et al., 1990).

Strain MYY812 was transformed with a genomic DNA library in plasmid YEp13 (Broach et al., 1979). Three plasmids (YEp13-1.1, YEp13-2.2, and YEp13-4.1) containing identical yeast DNA inserts were isolated and analyzed as described above.

Integrative Mapping

The *smm1* mutation was mapped to the *RSP5* locus by integrative transformation and genetic analysis. The integrating vector pRS305-RSP5 was linearized by digestion with MscI and transformed into strain MYY298. The resulting strain was sporulated, and a haploid spore with the *LEU2* gene integrated at the *RSP5* locus was identified (strain MYY820). This strain was crossed to *smm1* strain MYY808, the diploid was sporulated, and haploid progeny were scored for Leu^+ and growth at 37°C. No recombinants were identified from 28 tetrads, indicating that *smm1* mapped to within 1.7 cM of *RSP5*.

The *smm2* mutation was mapped to the *BUL1* locus by analyzing meiotic progeny resulting from a cross of strain MYY813 (*smm2*) to strain MYY826, which contained a disrupted copy of *bul1* (*bul1*::*LEU2*). Among 20 tetrads analyzed, all spores were temperature-sensitive, indicating no recombination between *smm2* and *bul1* and revealing genetic linkage of these two loci within 2.5 cM.

Plasmid Construction

Plasmids pRS316-RSP5 and pRS305-RSP5 were created by cloning the 4,031-bp XbaI-XhoI fragment containing the *RSP5* gene from plasmid YCp50-3.1 into the XbaI and XhoI sites of pRS316 (Sikorski and Hieter, 1989) and into the SalI and XbaI sites of pRS305 (Sikorski and Hieter, 1989), respectively.

Plasmid pBS-BUL1 was created by cloning the 5,456-bp NheI-SalI fragment from YEp13-2.2 into the SpeI and SalI sites of pBluescript KS^+ (Stragene). Plasmid pBS-Δbul1 was created by replacing the region of pBS-BUL1 between the outermost EcoRV sites with *LEU2* as follows. After digestion of pBS-BUL1 with EcoRV and treatment with calf intestinal phosphatase (CIP), the 5,834-bp vector backbone was isolated by gel purification. A 2,217-bp fragment containing the *LEU2* gene was isolated from plasmid YEp13 after digestion with SalI and XhoI and treatment with the Klenow fragment of DNA polymerase I and ligated into the pBS-BUL1 EcoRV-deleted backbone.

Plasmids pTER21, pTER22, pTER23, encoding mutant ubiquitin (K29R, K48R, and K63R mutations, respectively) under control of the *CUP1* promoter were described previously (Ellison and Hochstrasser, 1991; Arnason and Ellison, 1994). Plasmid pUb, which contains wild-type ubiquitin driven by the *CUP1* promoter, was created by replacing the BglII-SalI fragment from pTER22 with the BglII-SalI fragment from plasmid YEp105 (Ellison and Hochstrasser, 1991).

Plasmid pSB-smm1 was isolated by plasmid-mediated gap repair (Orr-Weaver et al., 1983), as described below. Plasmids pRS316-CA, pRS316-mdp1-1, pRS316-mdp1-13, and pRS316-mdp1-14 were generated by PCR-mediated site-directed mutagenesis and fragment-mediated gap repair of pRS316-RSP5, as described below. Plasmid pRS426-smm1 was created by replacing the 3,897-bp region between the BspEI and XhoI sites of pRS426-RSP5 with the corresponding fragment from pSB-smm1. Plasmid pRS316-smm1 was created by replacing the 3,897-bp BspEI-XhoI fragment of pRS316-RSP5 with the corresponding fragment from pRS426-smm1.

Mutational Analysis and Mutagenesis

The *smm1* mutation was shown to lie within *RSP5* by plasmid-mediated gap repair (Orr-Weaver et al., 1983). Plasmid pSB-H8 was digested with PvuII and ApaI. The resulting vector backbone (17,006 bp) was gel purified, redigested with the same enzyme combination, dephosphorylated with CIP, and transformed into MYY808. 24 independent Leu^+ isolates were tested for the ability to grow at 37°C. A plasmid, designated pSB-smm1, was isolated from one of the resulting Leu^+, temperature-sensitive clones. The DNA region of this plasmid between PvuII and ApaI was sequenced, and the only mutation found was a change of G to T at nucleotide 2258 of *RSP5*. The sequence of this region of the *RSP5* locus was also determined from genomic DNA isolated from MYY808 and MYY823 by asymmetric PCR (McCabe, 1990) as described previously (Fisk and Yaffe, 1997). For both strains, the only mutation found was G to T at nucleotide 2258 of *RSP5*, resulting in the substitution of valine for glycine at position 753 of Rsp5p (both numbers are relative to +1 of the *RSP5* open reading frame).

A diploid strain deleted for one copy of *RSP5* (strain MYY825) was derived from strain MYY298 by PCR-mediated gene disruption using the *HIS3* gene as a selectable marker as previously described (Berger and Yaffe, 1998). A strain deleted for *BUL1* was created by transforming the wild-type diploid strain MYY298 with plasmid pBS-ΔBUL1 which had been digested with BglII and BamHI. A Leu^+ transformant was then sporulated, and Leu^+ haploid spores of *MAT*a (MYY816) and *MAT*α (MYY817) genotypes were isolated. Gene disruptions were confirmed by PCR analysis.

The site-directed mutation C777A was created in *RSP5* by PCR in a manner similar to that described by Imhof and McDonnell (1996), and was cloned into pRS316-RSP5 by fragment-mediated gap repair as follows. Two separate PCR reactions were performed using pRS316-RSP5 as a template. The first reaction used the primer pair 5′-CTCACACAgcTTTTAACAGAG-3′ and 5′-GGCGAAGGGGGGATGTG-3′ (binds in the multicloning site of pRS316-RSP5, on the 3′ side of *RSP5*), and the second used the primer pair 5′-GACGAGGTCATTCAATGG-3′ and 5′-CTCTGTTAAAAgcTGTGTGAG-3′. The lowercase letters in these primer sequences indicate mutagenic nucleotides. The products of these two reactions, which overlap by 20 nucleotides, were combined and reamplified in the absence of primers to create a final PCR product containing the C777A mutation and spanning from nucleotide 2134 of *RSP5* across the pRS316 multicloning site on the 3′ side of *RSP5*. Plasmid pRS316-RSP5 was digested with MfeI and SacII and dephosphorylated by CIP treatment. The 7,576-bp vector backbone was gel purified and transformed into MYY823 together with the final PCR product. Sac II cuts pRS316-RSP5 outside of the yeast insert such that one end of the digested vector only has homology with the cotransformed PCR product. Therefore the only event that can repair the gapped plasmid is recombination with the PCR product to generate pRS316-CA. Transformation of MYY823 with digested vector alone yielded nine Ura^+ clones, whereas cotransformation of digested vector and the C777A-containing PCR product yielded 323 Ura^+ clones. All Ura^+ clones tested failed to grow at 37°C. Plasmids were isolated from these clones and digested with NlaIII to verify the presence of C777A, and with BsmAI to verify the absence of *smm1*. One of these clones was designated pRS316-CA, and sequenced to verify that C777A was the only nucleotide change.

The *RSP5 mdp1* mutations (Zoladek et al., 1997) were created in pRS316-RSP5 by the method described above, using the following mutagenic primers: *mdp1-1*, 5′AGTTGATTTGaCACAATACGT-3′, and 5′-ACGTATTGTGtCAAATCAACT-3′; *mdp1-13*, 5′-GATTATCGTGaTTACCAAGAG-3′, and 5′-CTCTTGGTAAtCACGATAATC-3′; *mdp1-14*, 5′-CCTGTCAACGaGTTTAAAGAT3′, and 5′-ATCTTTAAACtCGTTGACAGG-3′. Plasmids were isolated from yeast and sequenced to verify that the *mdp1* mutations were the only changes present.

Results

Isolation of Second-Site Suppressors of mdm1-252

To identify proteins interacting with Mdm1p to mediate mitochondrial distribution and morphology, second site suppressor analysis was performed using the *mdm1-252*

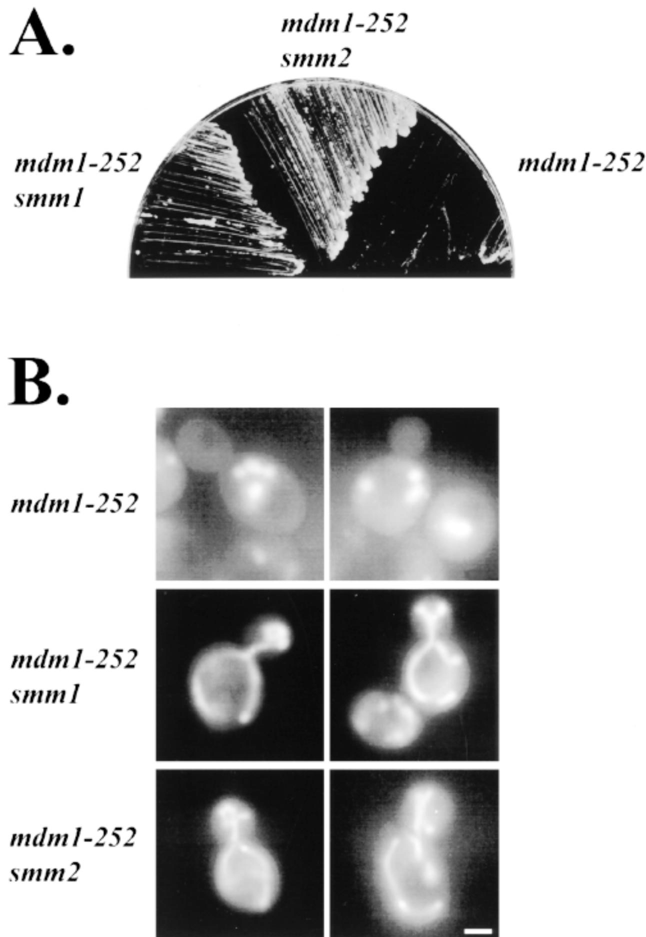

Figure 1. Suppression of *mdm1-252* defects by *smm1* and *smm2.* (A) Cells harboring the *mdm1-252* mutation (MYY721), or *mdm1-252* together with the *smm1* (MYY803) or *smm2* (MYY804) mutation were cultured on YPD medium for 48 h at 37°C. (B) Strains MYY721, MYY803, and MYY804 were grown on YPD at 23°C, incubated for 4 h at 37°C, and mitochondria were visualized by staining with DASPMI and fluorescence microscopy. Shown are two representative cells for each strain. Bar, 2 μm.

mutation. This mutation causes temperature-sensitive growth and defects in mitochondrial distribution and morphology, but does not cause abnormal nuclear inheritance (Fisk and Yaffe, 1997). Eight clones capable of growth at 37°C were identified from $\sim 5 \times 10^8$ cells. Genetic and microscopic analysis revealed that two of these strains possessed single, distinct mutations which suppressed both temperature-sensitive growth (Fig. 1 A) and mitochondrial distribution and morphology defects (Fig. 1 B) caused by *mdm1-252.* These suppressor mutations also conferred temperature-sensitive growth on cells when separated genetically from the original *mdm1252* lesion (Fig. 2). These new mutations, *smm1* and *smm2* (suppressor of Mdm1p-dependent mitochondrial inheritance defects), were found to be unlinked to *MDM1.*

The specificity of suppression by the *smm1* and *smm2* mutations was determined by crossing cells harboring these lesions to a collection of 10 otherwise isogenic strains containing different *mdm1* alleles (Fisk and Yaffe, 1997) and analyzing the phenotypes of haploid progeny harboring both the *mdm1* allele and the suppressor mutation. Suppression of *mdm1* by *smm1* was highly allele-specific: *smm1* failed to suppress the mutant phenotypes of any allele other than *mdm1-252.* In contrast, *smm2* partially suppressed the mutant phenotypes of several *mdm1* alleles including *mdm1-202*, *mdm1-204*, *mdm1-251*, and *mdm1-252.* Each of these *smm2*-suppressed *mdm1* mutations is a dominant allele (Fisk and Yaffe, 1997). Additionally, *smm2* displayed nonallelic noncomplementation with the recessive *mdm1* alleles: heterozygous *mdm1*/*MDM1 smm2*/*SMM2* diploids displayed temperature-sensitive growth.

smm1 Affects Mitochondrial Distribution and Morphology

Cells harboring the *smm1* or *smm2* mutation were analyzed microscopically to assess the possible effect of these lesions on mitochondrial distribution and morphology. At permissive temperature (23°C), mitochondrial distribution and morphology in *smm1* cells was indistinguishable from wild-type cells (Fig. 3 A). In contrast, cells harboring the *smm1* mutation displayed dramatic aberrations in mitochondrial distribution and morphology, similar to those caused by *mdm1-252*, after incubation at 37°C (Fig. 3 A). Indirect immunofluorescence microscopy further revealed that mitochondria in *smm1* cells at 37°C formed small round structures of uniform size which failed to enter buds in a large proportion of cells (Fig. 3 B). DAPI staining of *smm1* cells indicated that the *smm1* mutation was specific for mitochondrial inheritance, as there was no detectable defect in nuclear segregation (Fig. 3 B). Finally, there appeared to be no effect of *smm1* on the distribution or stability of Mdm1p cytoplasmic structures (Fig. 3 B). Therefore, *smm1* is a mitochondrial distribution and morphology (*mdm*) mutant with a phenotype very similar to that of *mdm1-252* cells. In contrast, *smm2* mutant cells displayed no defects in mitochondrial distribution or morphology at either permissive or nonpermissive temperatures (data not shown).

smm1 Is a Mutation in *RSP5*

To understand the mechanism of suppression of *mdm1-252*, *smm1* was cloned by complementation. Four plasmids which completely restored growth at 37°C to *smm1* cells were isolated. These plasmids also corrected defects in mitochondrial distribution and morphology. Restriction enzyme and nucleotide sequence analysis revealed that these plasmids contained overlapping inserts of yeast genomic DNA corresponding to a 5.6-kb region of chromosome V. Transformation of *smm1* cells with different DNA fragments derived from the genomic inserts localized complementing activity to *RSP5*, an essential gene encoding a ubiquitin-protein ligase containing a HECT domain (Huibregtse et al., 1995). Integrative mapping (as described in Materials and Methods) confirmed that the *smm1* mutation mapped to *RSP5.*

The *smm1* mutation was mapped within *RSP5* by plasmid-mediated gap repair, and the molecular identity of the mutation was determined by nucleotide sequencing of the

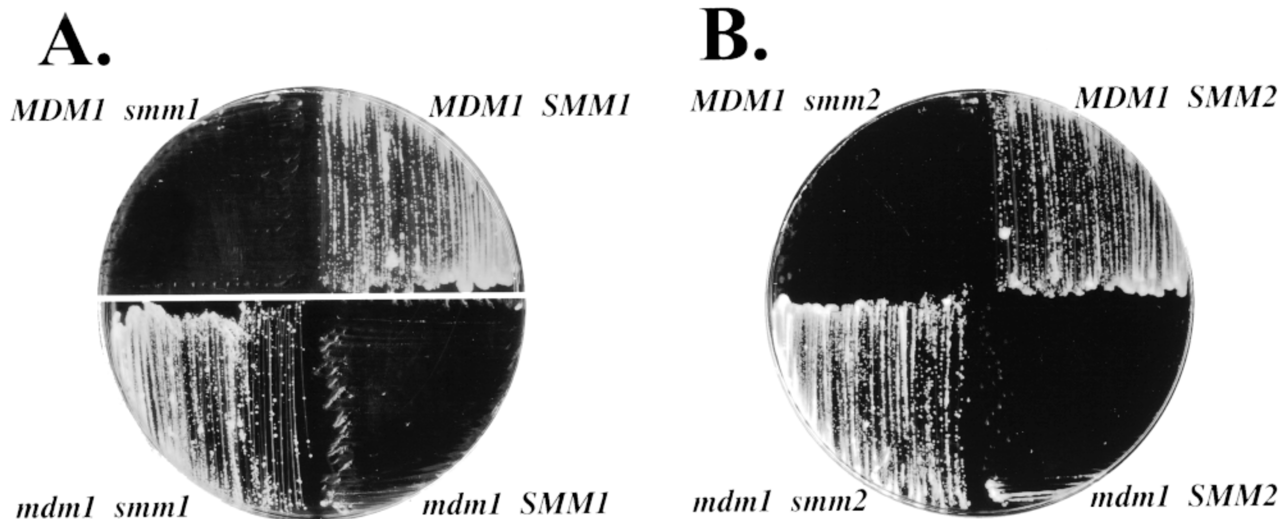

Figure 2. *smm1* and *smm2* confer temperature-sensitive growth. Strains MYY803 and MYY804 were backcrossed to MYY291 to separate *mdm1-252* from *smm1* and *smm2*, respectively. Spores of the indicated genotypes were plated on YPD medium and incubated 48 h at 37°C. (A) Growth at 37°C of spores from a cross of MYY803 and MYY291; (B) growth at 37°C of spores from a cross of MYY804 and MYY291.

appropriate region of the mutant gene. This analysis revealed a single change in the mutant gene, a transversion of G to T at nucleotide 2258. This mutation in the region of *RSP5* corresponding to the conserved HECT domain (Huibregtse et al., 1995) leads to a change of glycine-753 to valine.

smm2 Is a Mutation in *BUL1*

To investigate suppression by the *smm2* mutation, genomic plasmids that complemented the temperature-sensitive phenotype of *smm2* mutant cells were isolated. Three complementing plasmids contained the same genomic DNA insert encoding two genes, *RCE1* and *BUL1*, located on chromosome XIII. The complementing activity was mapped to *BUL1* by subcloning and retesting complementation in *smm2* cells. The isolated *BUL1* gene was shown to correspond to sequences from the *smm2* locus by integrative transformation and mapping. *BUL1* was previously shown to encode a 109-kD protein which binds to the ubiquitin-ligase Rsp5p (Yashiroda et al., 1996).

The *smm2* mutation did not cause defects in mitochondrial distribution or morphology (see above). To examine further a possible role for *BUL1* in mitochondrial inheritance, a *bul1*-null allele was created. Like the *smm2* mutant, cells deleted for *BUL1* (Δ*bul1*) displayed temperature-sensitive growth but no alteration in mitochondrial distribution or morphology at either permissive or nonpermissive temperature (data not shown). Additionally, the *bul1*-null mutation failed to suppress *mdm1-252*.

Ubiquitin Overexpression Influences Mitochondrial Inheritance

Previously, several mutations in the HECT domain of Rsp5p were shown to be suppressed by ubiquitin overexpression (Zoladek et al., 1997). To test whether the *smm1* mutation could be similarly suppressed, *smm1* mutant cells were transformed with plasmids encoding either wild-type or mutant ubiquitin expressed from the copper-inducible *CUP1* promoter. These cells were incubated at 37°C in the presence of 100 μM $CuSO_4$ (to induce high levels of ubiquitin) and examined by microscopy to assess effects on mitochondrial distribution and morphology. Overexpression of wild-type ubiquitin was found to suppress the mitochondrial morphology and distribution defects of *smm1* cells (Fig. 4 A). The specificity of this suppression was investigated by overexpression of versions of ubiquitin mutated in one of three critical lysine residues, K29R (UbK29R), K48R (UbK48R), or K63R (UbK63R). Overexpression of UbK29R or UbK48R suppressed the mitochondrial morphology and distribution defects, similar to suppression by wild-type ubiquitin (data not shown). However, the UbK63R mutant failed to suppress *smm1* (Fig. 4 A). No effect of ubiquitin overexpression was observed in *mdm1-252* cells at either permissive or nonpermissive temperatures (data not shown).

In control experiments, the effect of ubiquitin overexpression was examined in wild-type cells. Overexpression of wild-type ubiquitin (Fig. 4 A), UbK29R, or UbK48R had no apparent effect on mitochondrial distribution or morphology. However, expression of UbK63R perturbed mitochondrial inheritance in wild-type cells (Fig. 4, A and B). A quantitative analysis of this effect revealed that ~30% of MYY290 cells expressing UbK63R displayed buds devoid of mitochondria, a frequency similar to that seen in MYY823 (*smm1*) cells (Fig. 4 A). In addition, these cells possessed empty buds of medium to large size and displayed pronounced mitochondrial aggregations (Fig. 4 B). The effect of ubiquitin overexpression was identical whether copper induction was simultaneous with temperature shift or if ubiquitin overexpression was induced for 2 h before temperature shift (data not shown). These results suggest that the formation of polyubiquitin

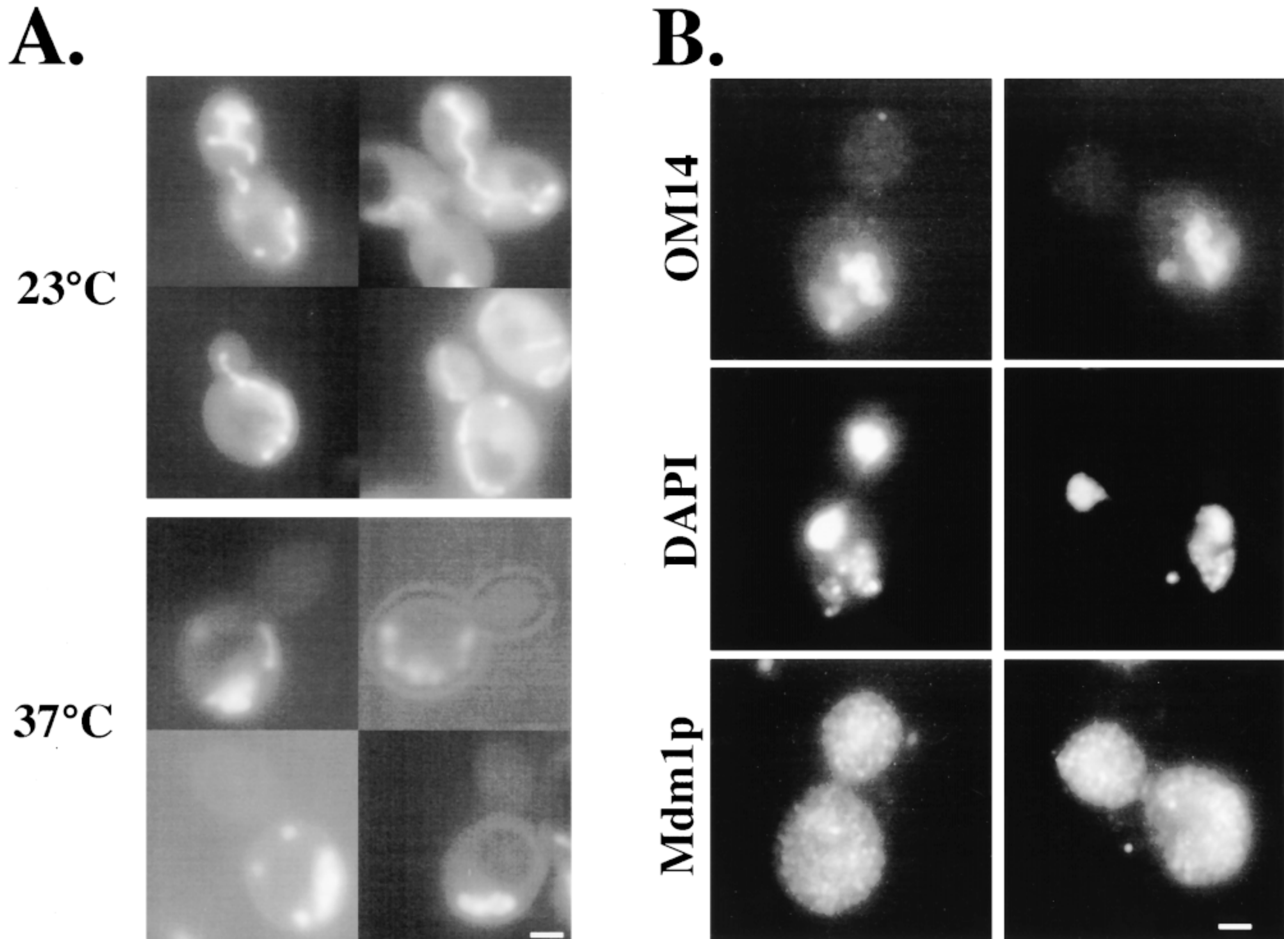

Figure 3. The *smm1* mutation causes mitochondrial inheritance defects. Strain MYY809 (*smm1*) was cultured on YPD medium at 23°C and then incubated for 4 h at 23°C or 37°C. (A) Cells were stained with DASPMI to visualize mitochondria. Bar, 2 μm. (B) Cells which had been shifted to 37°C for 4 h were fixed with formaldehyde and processed for indirect immunofluorescence. Mitochondria were detected with anti-OM14 followed by fluorescein-conjugated donkey anti–mouse IgG (top). Mitochondrial and nuclear DNAs were stained with DAPI (middle). Mdm1p was detected with affinity-purified anti-Mdm1p followed by rhodamine-conjugated donkey anti–rabbit IgG (bottom). Shown are two representative cells. Bar, 2 μm.

chains linked via lysine-63 is essential for mitochondrial inheritance.

smm1 Is Distinct from Previously Identified *RSP5* Mutations

Several other temperature-sensitive mutations in *RSP*5, the *mdp1* mutations, were shown to map to the HECT domain (Zoladek et al., 1997). To determine if these mutations behave like *smm1* with respect to mitochondrial inheritance, the effect of three previously described lesions, *mdp1-1*, *mdp1-13*, and *mdp1-14*, on suppression of *mdm1-252* and on mitochondrial distribution in the MYY290 genetic background was examined. As expected, each of the mutations conferred a temperature-sensitive growth phenotype on otherwise wild-type cells, but none of the three *mdp1* mutations suppressed the *mdm1-252* phenotypes (data not shown).

To determine whether the *mdp1* mutations affected mitochondrial distribution and morphology, cells were incubated at 37°C and examined microscopically after staining with DASPMI. *mdp1-1* caused a modest defect in mitochondrial distribution and morphology, although considerably less than that caused by *smm1* (Fig. 5). Neither *mdp1-13* nor *mdp1-14* caused any defect in mitochondrial inheritance or morphology relative to wild-type *RSP5* (Fig. 5). These results indicate that the *smm1* mutation confers properties unique from those caused by previously described mutations in *RSP5.* These findings are largely consistent with a previous study (Zoladek et al., 1995) which found that the *mdp1* mutation in a different strain background caused no defects in mitochondrial distribution or morphology.

Ubiquitination Is Required for Mitochondrial Inheritance

The *smm1* mutation lies in the HECT domain of Rsp5p, suggesting that the mutation may affect ubiquitin ligase activity or substrate specificity. To test whether the ubiquitin ligase activity of Rsp5p is required for mitochondrial inheritance, a mutation in a key residue of the active site

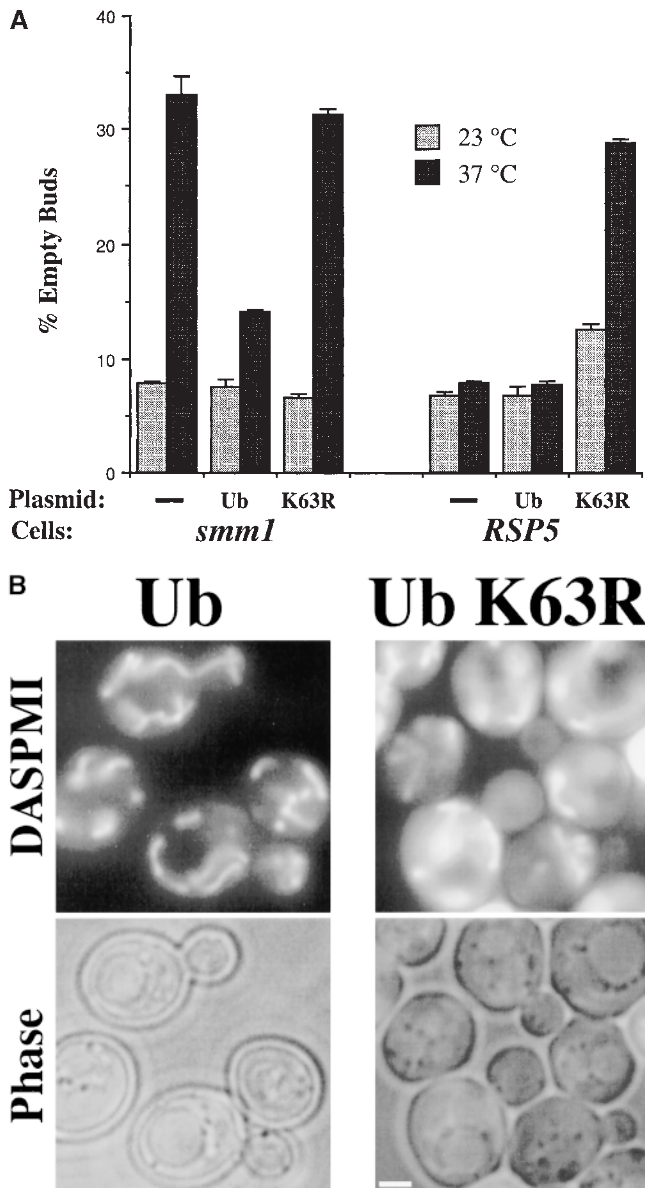

Figure 4. Ubiquitin overexpression affects mitochondrial distribution and morphology. *smm1* mutant cells (MYY823) and wild-type cells (MYY290) which harbored no plasmid (−), plasmid pUb (Ub), or plasmid pTER23 (K63R) were cultured at 23°C on minimal medium lacking uracil. $CuSO_4$ was added to 100 μM, and cells were incubated for 2 h at 23°C or 37°C. (A) Cells were fixed, stained with DAPI, and examined by fluorescence microscopy. Budded cells were scored as possessing empty buds if no mitochondrial DNA nucleoids were observed in the bud. Percentages of empty buds were determined for duplicate samples with at least 300 budded cells counted per sample. (B) Wild-type cells (MYY290) transformed with pUb (Ub) or pTER23 (Ub K63R) were incubated at 37°C for 2 h in the presence of 100 μm $CuSO_4$ and stained with DASPMI. Cells were examined by fluorescence (DASPMI) or phase-contrast (Phase) microscopy. Bar, 2 μm.

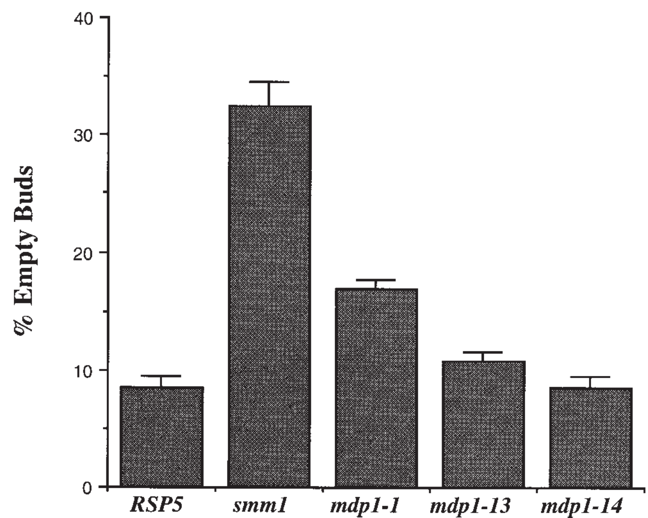

Figure 5. Differential effects of *rsp5* mutations on mitochondrial inheritance. Haploid *rsp5*-null strains harboring plasmids containing either wild-type *RSP5* (MYY826) or mutant forms of *rsp5* with the *smm1* (MYY829), *mdp1-1* (MYY832), *mdp1-13* (MYY833), or *mdp1-14* (MYY834) mutation were cultured on minimal medium lacking uracil at 23°C. Cells were incubated at 37°C for 2 h, stained with DASPMI, and examined by fluorescence microscopy. Percentages of empty buds were determined for budded cells in duplicate samples with at least 200 cells counted per sample.

of Rsp5p was created. The conversion of cysteine-777 to alanine (C777A) was shown previously to destroy the ability of Rsp5p to form a covalent intermediate with ubiquitin, thereby destroying its ubiquitin ligase activity (Huibregtse et al., 1995). The C777A mutation was generated in a plasmid-borne copy of *RSP5*, and the plasmid was transformed into *smm1* cells. The C777A mutant *rsp5* failed to complement either the *smm1* temperature-sensitive growth phenotype (Fig. 6 A) or the *smm1* defects in mitochondrial distribution and morphology (Fig. 6 B). These results demonstrate that the ubiquitin ligase activity of Rsp5p is essential for its function in mitochondrial inheritance.

To further evaluate the requirement of ubiquitination for mitochondrial inheritance, mitochondrial distribution and morphology were examined in cells defective for ubiquitin-conjugating enzymes (E2-type enzymes). *S. cerevisiae* possesses genes encoding more than a dozen different E2 proteins, but much of the cytoplasmic ubiquitination activity depends on two proteins with redundant specificity, Ubc4p and Ubc5p (Seufert and Jentsch, 1990; Seufert et al., 1990). Strains deleted for *UBC4*, *UBC5*, or *UBC1* (or combinations of two of these genes) were examined by fluorescence microscopy after mitochondrial staining. Cells with null mutations in both *UBC4* and *UBC5* displayed aggregated mitochondria and daughter buds devoid of mitochondria (Fig. 7). Both mutant traits were more prevalent after incubation of cells at 37°C, and a quantitation of this effect after 4 h at 37°C revealed 86% of cells with aggregated mitochondria and 52% of budded cells with empty daughter buds. These mutant traits were not apparent in cells with only *ubc4* or *ubc5* mutations, nor were they found in *ubc1* or *ubc1 ubc4* mutants (data not shown). In addition, the inheritance and distribution of nuclei were normal in the *ubc4 ubc5* mutant cells (data not shown). These results suggest that the ubiquitin-conjugat-

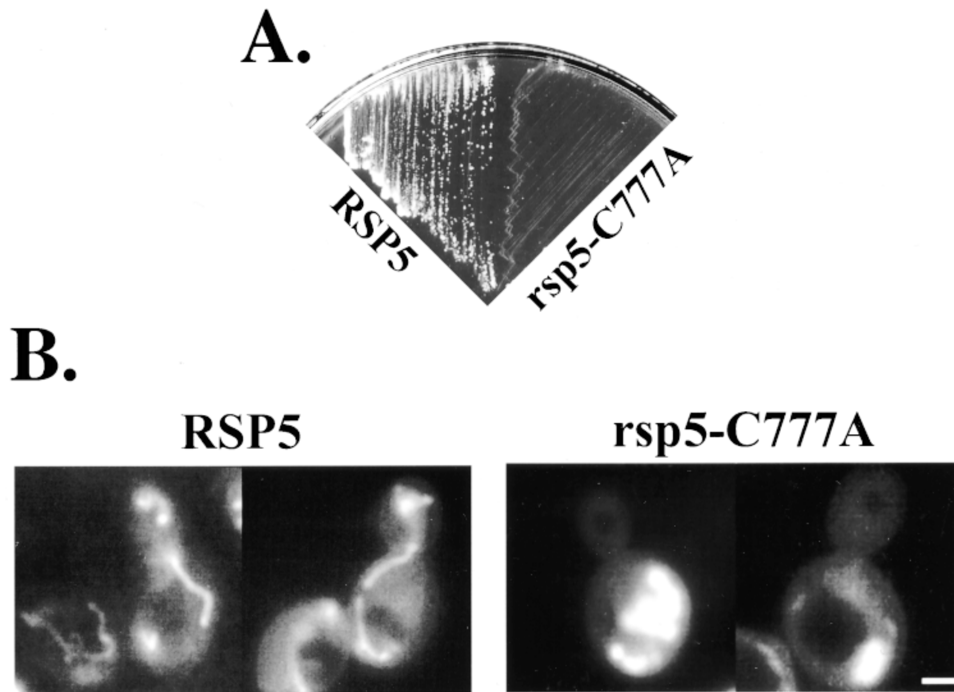

Figure 6. Ubiquitin ligase activity of Rsp5p is required for mitochondrial inheritance. Cells harboring the *smm1* mutation (MYY823) were transformed with centromere-based plasmids encoding wild-type *RSP5* (pRS316-RSP5) or *rsp5* bearing the C777A mutation (pRS316-CA). (A) Transformants were incubated on YPD medium at 37°C for 48 h. (B) Cells were cultured in minimal medium lacking uracil, shifted to 37°C for 4 h, stained with DASPMI, and examined by fluorescence microscopy. Bar, 2 μm.

ing enzymes Ubc4p and Ubc5p mediate ubiquitination reactions essential for mitochondrial inheritance.

Discussion

We have uncovered a novel role for protein ubiquitination in mitochondrial inheritance. This role was revealed through the characterization of *smm1*, a mutation that suppresses the mitochondrial distribution and morphology defects caused by *mdm1-252.* Six key findings support this new function for ubiquitination. First, *smm1* mapped to *RSP5*, an essential gene encoding a ubiquitin-protein ligase. Second, the *smm1* mutation alone conferred conditional defects in mitochondrial distribution and morphology. Third, the defects caused by *smm1* were complemented by wild-type Rsp5p but not by mutant Rsp5p lacking ubiquitin ligase activity. Fourth, *smm2*, a second mutation suppressing *mdm1-252*, mapped to *BUL1*, a gene encoding a protein that binds to Rsp5p and facilitates its activity (Yashiroda et al., 1996). Fifth, overexpression of a mutant form of ubiquitin which blocks elongation of certain polyubiquitin chains also caused aberrant mitochondrial distribution and morphology in wild-type cells. Finally, depletion of two ubiquitin-conjugating enzymes, Ubc4p and Ubc5p, caused defective mitochondrial morphology and inheritance.

Protein ubiquitination has been found to play a key role in a variety of cellular functions. Prominent among these roles is the ubiquitin-mediated targeting of cytosolic proteins for degradation by the proteosome (Hochstrasser, 1996; Pickart, 1997). Similarly, the proteosome-dependent turnover of several membrane proteins of the endoplasmic reticulum is initiated by their ubiquitination (Brodsky and McCracken, 1997; Hampton and Bhakta, 1997). Ubiquitination of plasma membrane proteins including the yeast Fur4p (Galan et al., 1996), Ste2p (Hicke and Riezman,

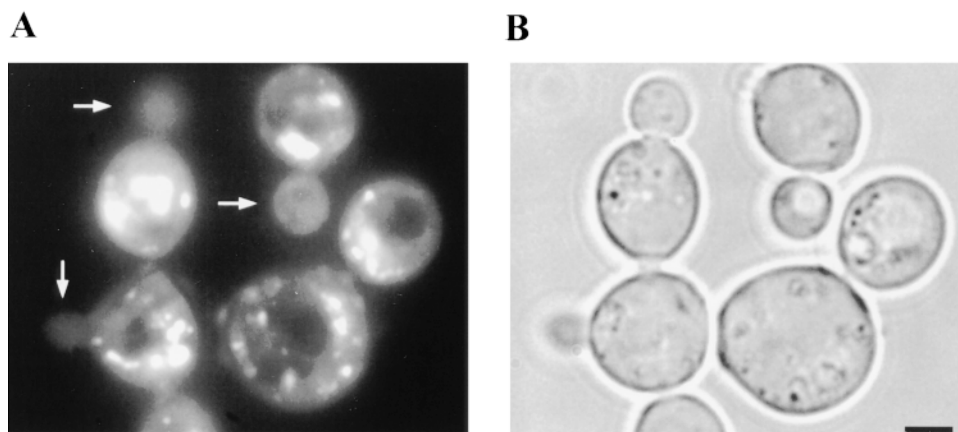

Figure 7. Mutations in ubiquitin-conjugating enzymes cause defects in mitochondrial morphology and inheritance. Cells harboring null mutations in both *ubc4* and *ubc5* (LHY21) were cultured at 23°C on YPD medium, incubated at 37°C for 4 h, stained with DASPMI, and examined by fluorescence microscopy. Arrows indicate daughter buds without mitochondria. Bar, 2 μm.

1996), and Gap1p (Springael and Andre, 1998) has been shown to trigger their endocytosis and transport to the vacuole where they are subsequently degraded in a process independent of the proteosome. Protein ubiquitination has also been found to function in as yet poorly defined roles in the import of certain proteins into peroxisomes (Wiebel and Kunau, 1992; Crane et al., 1994) and mitochondria (Zhuang and McCauley, 1989; Zoladek et al., 1997). Additionally, recent studies have revealed that the conjugation of protein targets with ubiquitin-like proteins is an important feature of nuclear protein import (Mahajan et al., 1997) and cell cycle regulation (Liakopoulos et al., 1998). Our identification of a role for protein ubiquitination in mitochondrial inheritance represents a novel cellular function for this consequential covalent modification.

The *smm1* and *mdm1-252* mutations display highly specific reciprocal suppression. *smm1* did not suppress any other *mdm1* allele, nor was *mdm1-252* or any other *mdm1* allele suppressed by other *rsp5* mutations. This specificity and reciprocity suggests that Rsp5p and Mdm1p interact directly to effect normal mitochondrial inheritance. The results suggest further that the *smm1* and *mdm1-252* mutations interfere individually with this interaction but that the combination of the two lesions restores functionality. Similar reciprocal suppression has been demonstrated for specific mutations in actin and actin-binding protein Sac6p in *S. cerevisiae* (Adams and Botstein, 1989; Adams et al., 1989). Future studies will evaluate the possible direct binding or transient interactions of Rsp5p and Mdm1p.

The mapping of the *smm2* mutation to *BUL1* further supports a role for ubiquitination in mitochondrial inheritance. The *BUL1* product, Bul1p, was previously identified as a protein that binds to Rsp5p (Yashiroda et al., 1996), and it has been proposed to function as a cofactor, modulating the activity or specificity of the ubiquitin ligase. A similar role is played by the E6 protein of human papilloma virus in the ubiquitination of p53 (Huibregtse et al., 1991). Although *smm2* suppressed *mdm1-252*, it also displayed genetic interactions with several other *mdm1* alleles, indicating that Bul1p may not interact directly with Mdm1p. Furthermore, neither *smm2* nor the *bul1*-null mutation caused any apparent defect in mitochondrial inheritance, indicating that Bul1p is not normally involved in this process. One hypothesis consistent with these observations is that one of three Bul1p homologues in *S. cerevisiae*, Yml111p, Ynr068p, or Ynr069p, may be required for mitochondrial inheritance, and the *smm2* mutation might allow Bul1p to supplement or interfere with the activity of its homologue.

The target of Rsp5p-mediated ubiquitination associated with mitochondrial inheritance is unknown. Rsp5p was previously shown to ubiquitinate a variety of cellular substrates including the large subunit of RNA polymerase II (Huibregtse et al., 1997) as well as the plasma membrane proteins Fur4p (Galan et al., 1996) and Gap1p (Springael and Andre, 1998). Mdm1p appeared to be a reasonable candidate for ubiquitination by Rsp5p, but no evidence of such ubiquitination was obtained. In particular, immunoblot analysis of cellular proteins separated by two-dimensional polyacrylamide gel electrophoresis failed to reveal either ubiquitin associated with Mdm1p or higher molecular weight forms of Mdm1p that might represent ubiquitinated species (data not shown). Other candidates for ubiquitination include proteins integral or bound to the mitochondrial outer membrane. The identification of the relevant Rsp5p substrates may emerge from analysis of proteins ubiquitinated in wild-type cells but not in *smm1* or *mdm1-252* mutant cells at 37°C.

What might be the role of ubiquitination in mitochondrial inheritance? One possibility is that the ubiquitination of one or more key proteins initiates changes in the interaction of mitochondria with Mdm1p structures. In this model, Rsp5p might first bind to Mdm1p and then ubiquitinate a nearby target protein. The consequence of this activity could be either to promote or diminish an interaction between the target protein and Mdm1p. For example, ubiquitination might promote an association of mitochondria with Mdm1p structures as a critical step in the mitochondrial distribution process. Alternatively, ubiquitination of a mitochondrial surface protein might facilitate its dissociation from Mdm1p structures to mobilize mitochondria. The direct effect of ubiquitination could be to target the ubiquitinated substrate for degradation or, alternatively, ubiquitination might function like other types of covalent modifications to alter the activity or structure of the target protein. The dependence of mitochondrial inheritance on lysine-63 (K63) of ubiquitin may provide a clue to the fate of the ubiquitinated target protein. Fur4p modification by Rsp5p-dependent addition of K63-linked polyubiquitin chains leads to the internalization of this protein from the plasma membrane in a proteosome-independent event (Galan and Haguenauer-Tsapis, 1997). In contrast, the addition of polyubiquitin chains linked through lysine-48 leads to the proteosome-dependent degradation of the MAT $\alpha 2$ transcriptional regulator (Hochstrasser et al., 1991). Because Rsp5p-dependent formation of K63-linked chains appears to be required for mitochondrial inheritance, ubiquitination may play a proteosome-independent role in mitochondrial inheritance. The identification of the relevant substrates of ubiquitination and a characterization of these proteins' molecular interactions should uncover biochemical details of the function of ubiquitination in mitochondrial inheritance.

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